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Cassette mutagenesis: an efficient method for generation of multiple mutations at defined sites

(Recombinant DNA; oligonucleotide directed mutagenesis; protein engineering; subtilisin; *Escherichia coli*)

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SUMMARY

A method is described for the efficient insertion of mutagenic oligodeoxynucleotide cassettes which allow saturation of a target amino acid codon with multiple mutations. Restriction sites are introduced by oligonucleotide-directed mutagenesis procedures to flank closely the target codon in the plasmid containing the gene. The restriction sites to be introduced are chosen based on their uniqueness to the plasmid, proximity to the target codon and conservation of the final amino acid coding sequence. The flanking restriction sites in the plasmid are digested with the cognate restriction enzymes, and short synthetic duplex DNA cassettes (10–25 bp) are inserted. The mutagenic cassette is designed to restore fully the wild-type coding sequence, except over the target codon, and to eliminate one or both restriction sites. Elimination of a restriction site facilitates selection of clones containing the mutagenic oligodeoxynucleotide cassette. To make the cassettes, single-stranded oligodeoxynucleotides and their complements are synthesized in separate pools containing different codons over the target. This method has been successfully applied to generate 19 amino acid substitutions at position 222 in the subtilisin protein sequence.

INTRODUCTION

Oligodeoxynucleotide-directed mutagenesis (Zoller and Smith, 1982; Dalbadie-McFarland et al., 1982; Wallace et al., 1981; Hutchinson et al., 1978; Razin et al., 1978) provides a general method to allow replacement of any amino acid codon with another in a cloned gene. This is a powerful approach, permitting one to alter specifically the structure and thereby the function of an expressed protein. It is, however, often difficult to predict (even with a three-dimensional protein structure) which amino acid substitution results in a new or altered function of the protein. In such a case it is necessary to gain an empirical data base by characterizing many amino acid substitutions at the target site.

We have recently cloned the gene for *Bacillus amyloliquefaciens* subtilisin (Wells et al., 1983). Adjacent to the active-site serine residue is a methionine residue at position 222, which has been implicated as being sensitive to oxidation with resultant inactivation of the enzyme (Stauffer and Etson, 1969). The three-dimensional structure is known for subtilisin (Wright et al., 1969; Drenth et al., 1972); yet it is not

Abbreviations: bp, base pairs; BSA, bovine serum albumin; Cm, chloramphenicol; DTT, dithiothreitol; Δ, deletion; SDS, sodium dodecyl sulfate.

obvious from molecular modeling which residue would be the optimal substitute for the methionine to allow retention of enzyme activity and improve oxidative stability. To simplify the task of generating multiple mutations at a specific site we have employed a strategy referred to as cassette mutagenesis. Here we report on the construction of 19 different mutants at codon-222 using cassette mutagenesis. This method should be generally applicable to specific or random alteration of protein coding sequences over a defined sequence range.

MATERIALS AND METHODS

(a) Enzymes, media, transformation, and oligodeoxynucleotides

*Pst*I, *Bam*HI, *Eco*RI and T4 DNA ligase were from New England Biolabs. *Kpn*I and T4 polynucleotide kinase were from Bethesda Research Laboratories, and *Escherichia coli* DNA polymerase I large fragment (Klenow) was from Boehringer-Mannheim. LB media and plates (Miller, 1972) contained 12.5 μg Cm/ml unless otherwise stated. Transformation of *E. coli* 294 *rec*$^+$ cells was carried out using the $CaCl_2$ method (Mandel and Higa, 1970). Oligodeoxynucleotides were synthesized singly or in pools, as indicated, by addition of monomers and presynthesized trimers (or trimer mixtures) using phosphotriester chemistry (Crea and Horn, 1980), except that mesitylene nitrotriazole was used as a condensing agent. Oligodeoxynucleotide pools were synthesized by adding an equal mixture of five indicated trimers at the position of the target codon (see Figs. 1 and 2). Oligodeoxynucleotides were purified by polyacrylamide gel electrophoresis.

(b) Site-specific mutagenesis

The subtilisin gene is entirely contained on a 1.5-kb *Eco*RI-*Bam*HI fragment from the plasmid pS4-5 (Wells et al., 1983). This DNA fragment was ligated into phage M13mp9 (Sanger et al., 1980; Messing et al., 1981; Messing and Vieira, 1982), and single-stranded recombinant phage DNA (M13mp9SUBT) was prepared. An oligodeoxynucleotide was synthesized having the sequence 5′-GTACAACGGTACCTCACGCACGCTGCAGGAGCGGCTGC-3′ (38–mer). This was designed to create a *Kpn*I site 5′ to the met-222 codon and a *Pst*I site 3′ to the met-222 codon (see Figs. 1 and 2). The oligomer was [^{32}P]phosphorylated by incubation with [γ-^{32}P]ATP and T4 polynucleotide kinase followed by a chase with nonradioactive ATP to allow complete phosphorylation of the primer. The kinase was inactivated by heating to 68°C for 15 min. The annealing reaction, modified from Norris et al. (1983), contained 5 μl of mutagenic [^{32}P]primer (approx. 3 μM), approx. 1 μg M13mp9SUBT template, 1 μl of 1 μM M13 sequencing primer (17-mer), 2.5 μl of 10 × ligase buffer (0.3 M Tris·HCl pH 8, 40 mM $MgCl_2$, 12 mM EDTA, 10 mM DTT, 0.5 mg/ml BSA). The mixture was heated to 68°C for 10 min and cooled 10 min at room temperature. To the annealing mixture was added 3.6 μl containing 0.25 mM of each deoxynucleotide, 1.25 μl of 10 mM ATP, 1 μl ligase (4 units) and 1 μl Klenow (5 units), so the final volume was 25 μl. After incubation for 2 h at 14°C, the enzymes were inactivated by heating to 68°C for 20 min. The reaction mixture was digested with *Bam*HI + *Eco*RI, and an aliquot was run on a 6% polyacrylamide gel.

Autoradiography of this gel confirmed that the mutagenic [^{32}P]primer had indeed been incorporated into the *Eco*RI-*Bam*HI subtilisin gene fragment. The remainder of the digest was extracted once with a 1:1 (v:v) phenol–chloroform mixture, once with chloroform, and precipitated with ethanol. The DNA pellet was washed with 70% ethanol, lyophilized and taken up in 20 μl total of ligase buffer containing 0.5 mM ATP, 6 units T4 DNA ligase, and 0.5 μg pBS42 that had been cut with *Bam*HI + *Eco*RI and purified on a polyacrylamide gel as above. pBS42 is an *E. coli-B. subtilis* shuttle vector which contains a Cm resistance gene (*cat*) and convenient *Bam*HI and *Eco*RI cloning sites (Band and Henner, 1984). Ligation was performed overnight at 14°C, and the DNA was transformed into *E. coli*. The pool of transformants were grown out in LB + Cm, and plasmid DNA was prepared from this culture by the alkaline/SDS lysis method (Birnboim and Doly, 1979). Analysis of the DNA showed that 30–50% of the molecules contained the expected *Kpn*I site programmed by the mutagenic primer. The DNA was transformed a second time

into *E. coli* to clone plasmids containing the new *Kpn*I site. Of 16 transformants, six were found to contain the expected *Kpn*I site.

Further restriction analysis of one of these transformants (pΔ222) confirmed the presence and location of both the expected *Kpn*I and *Pst*I sites. Gapped pΔ222 was prepared by exhaustive *Kpn*I digestion, ethanol precipitation, and subsequently digestion with *Pst*I (approx. 400 units per 40 μg pΔ222 for 1.5 h at 37°C). The linearized pΔ222 was purified by polyacrylamide gel electrophoresis followed by electroelution of the vector band (Maniatis et al., 1982).

(c) Ligation of oligodeoxynucleotide pools

Complementary oligodeoxynucleotide pools (approx. 10 μM) which were not 5′-phosphorylated, were annealed in ligase buffer (20 μl total) by heating for 5 min at 68°C and then cooling for 15 min at room temperature. Individual pools of oligodeoxynucleotides (called A to D in Table I) at final concentrations of 1 μM were mixed with approx. 0.2 μg of *Kpn*I + *Pst*I-cut pΔ222 and ligated in a total volume of 20 μl overnight at 14°C. Then 25 μl of 10 mM Tris · HCl pH 8, 1 mM EDTA was added, and the mixture was reannealed to avoid inserts of linker concatemers by heating to 68°C and cooling to room temperature. The DNAs from the ligation mixtures were transformed (each pool separately) into *E. coli* 294 *rec*$^+$ cells. A small aliquot from each transformation mixture was plated to determine the number of independent transformants and the rest (200–400 transformants) cultured in 4 ml of LB + Cm. DNA was prepared from each transformation pool (A–D) and digested with *Kpn*I to eliminate pΔ222 vector contamination. The *Kpn*I-digested DNA was used to retransform *E. coli*, and the mixture was plated to isolate individual colonies from each pool. DNA was prepared from 24 to 26 transformants per pool for direct plasmid sequencing (Chen and Seeburg, 1985). Cultures of 5 ml were grown 16–20 h at 37°C, and DNA was prepared by a modification of the alkaline/SDS lysis procedure (Birnboim and Doly, 1979). This procedure was modified by addition of a phenol/chloroform extraction after lysis and a second phenol/chloroform extraction following a 30-min incubation with 10 μg of DNase-free RNase. The DNA was finally precipitated with ethanol, washed with 70% ethanol, lyophilized, and taken up in 20 μl of 10mM Tris·HCl pH 8, 1 mM EDTA. After alkaline denaturation of the plasmid and neutralization with NH_4·acetate, the DNA was precipitated with ethanol and dissolved in buffer for nucleotide sequencing. A synthetic oligodeoxynucleotide primer having the sequence 5′-GAGCTTGATGT-CATGGC-3′ which ends at codon-200 was used to prime the dideoxy sequencing reaction for analysis of codon-222.

RESULTS

(a) Construction of codon-222 mutants

The overall strategy of the cassette mutagenesis method is outlined in Fig. 1. The DNA sequence flanking codon-222 was searched to locate sequences which nearly matched a recognition sequence for a restriction enzyme not found in the wild-type subtilisin gene sequence (see DISCUSSION). As shown in Fig. 2, *Kpn*I and *Pst*I sites and a 10-bp deletion were introduced by M13 mutagenesis yielding pΔ222. Although pΔ222 could be digested to completion (> 98%) by either *Kpn*I or *Pst*I separately, exhaustive double digestion was incomplete (< < 50%). This may have resulted from the fact that these sites were so close (10 bp) that digestion by *Kpn*I allowed "breathing" of the DNA in the vicinity of the *Pst*I site, thus inhibiting subsequent *Pst*I digestion. Fortunately, contamination of pΔ222 in the final pool of transformants was readily eliminated. First, the oligodeoxynucleotide cassettes were *not* phosphorylated prior to ligation, and they were present in large excess (approx. 300×) over the free pΔ222 vector ends. Thus, circularization of single-cut vector was poisoned by ligation of the oligodeoxynucleotide. This was evidenced by the fact that the numbers of transformants from ligations in the absence and presence of oligodeoxynucleotide cassettes were 5000 and 1000, respectively.

Since insertion of an oligodeoxynucleotide cassette eliminates the *Kpn*I site, plasmid DNA that was isolated from the total pool of transformants could be analyzed for the continued presence of pΔ222 vector by *Kpn*I restriction analysis. Altogether, 30–50% of the plasmids from ligations with oligodeoxynucleotides still contained a *Kpn*I site (not shown). The residual pΔ222 contamination was es-

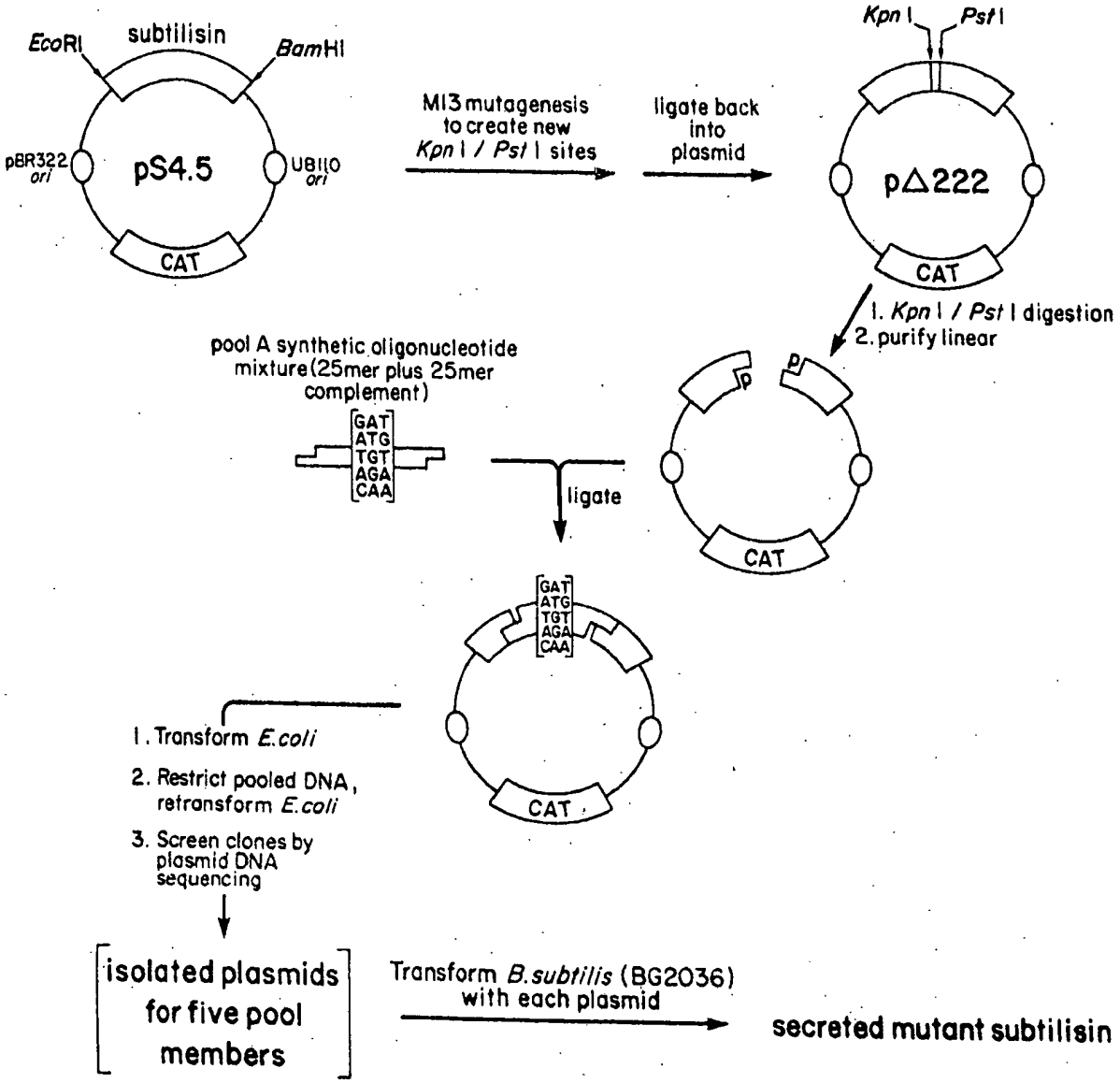

Fig. 1. The cassette mutagenesis method. The *Eco*RI-*Bam*HI fragment from pS4.5 containing the *B. amyloliquefaciens* subtilisin gene was cloned into M13mp9. Site-directed mutagenesis was performed to create unique *Kpn*I and *Pst*I sites closely flanking codon-222. The mutagenized *Eco*RI-*Bam*HI fragment was cloned back into pBS42 and screened for the *Kpn*I and *Pst*I sites. The desired mutant plasmid, pΔ222, was restricted with *Kpn*I and *Pst*I and the linearized plasmid purified. This was ligated in separate reactions with the four pools (A–D) of mutagenic oligodeoxynucleotide cassettes. Following transformation of *E. coli*, each pool of DNA was isolated and restricted with *Kpn*I to destroy any pΔ222 not containing a cassette. *Kpn*I restricted DNA was used to transform *E. coli*, and plasmid DNA from isolated colonies was screened for codon-222 mutants by single-track nucleotide sequencing. The isolated mutant plasmid was used to transform the protease-deficient mutant of *B. subtilis*, BG2036 (Yang et al., 1984), to produce secreted mutant subtilisin.

sentially eliminated by a second transformation with a *Kpn*I digested pool of plasmid DNA.

(b) Analysis of codon-222 mutants

The nucleotide sequence of first-round transformants described above was occasionally heterogeneous. This suggested that some clones were transformed with oligodeoxynucleotide cassette heteroduplexes that did not segregate. Thus, transformants were selected for analysis from the retransformation with *Kpn*I-digested DNA to ensure a homogeneous plasmid population in each clone. The oligodeoxynucleotide cassettes were organized into four pools of

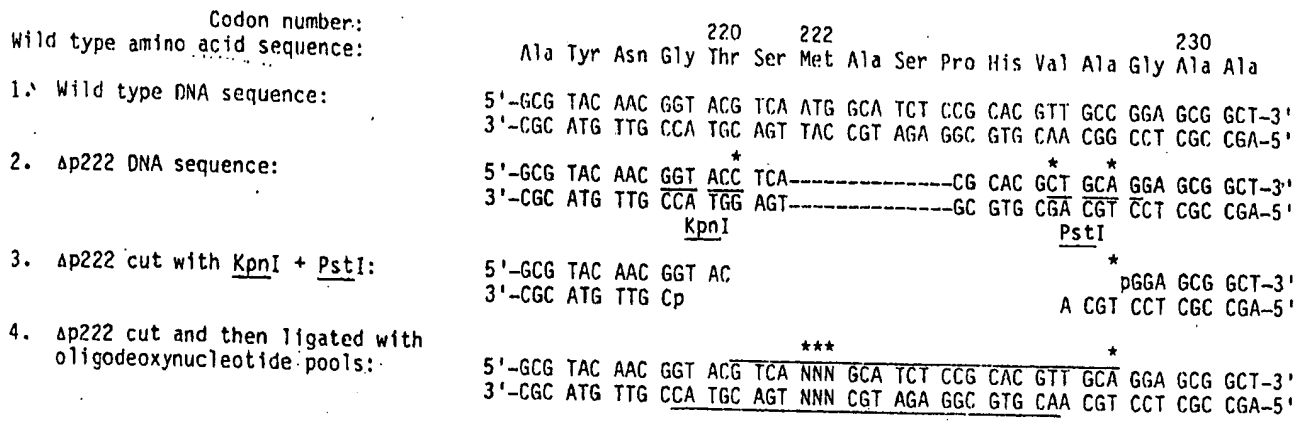

Fig. 2. The cassette mutagenesis strategy. Line 1: the wild-type *B. amyloliquefaciens* subtilisin-coding DNA sequence. Line 2: 38-mer mutagenic primer (see MATERIALS AND METHODS, section **b**) directed a 10-bp deletion and the introduction of *Kpn*I and *Pst*I sites (underlined). These sites were created by single base pair mutations shown by the asterisks. Line 3: pΔ222 as cut sequentially with *Kpn*I and *Pst*I. Line 4: synthetic oligodeoxynucleotide cassettes in pools containing five different codons-222 ligated into the *Kpn*I/*Pst*I gap; this recreated the wild-type coding sequence (see lines) except over codon-222, and eliminated the flanking restriction sites. Asterisks represent mutations in the subtilisin gene sequence.

five codons each (see Table I). The pools were organized so that a single sequencing track could distinguish members in a pool. A representative single-track sequence analysis is shown in Fig. 3. Of 30 codon-222 predictions made by single-track analysis, 29 were confirmed by four-track analysis. The frequency of codon-222 mutants obtained from each pool of transformants is shown in Table I. The fact that certain mutants appear more frequently than others (e.g., Cys vs. Gln in pool A) suggests that either the oligodeoxynucleotide pools are biased or that there is a biological bias. The latter is unlikely, in that bias was observed against appearance of the wild-type codon (met-222).

Unexpected mutants appeared at a frequency of about 30%. About one-fourth of these mutants were deletions that appeared inside the region of the oligonucleotide and usually at the point of *Pst*I ligation. About three-fourths of the unexpected mutants were double mutants, which often appeared as codon changes next to codon-222 (e.g., TTA, TCT, or TCG instead of TCA at codon 221). Double mutants often contained codon changes at the points of ligation (e.g., GTC instead of GTT at 226 or ATG instead of ACG at codon 220). Certain 222 codons had a disproportionate number of deletion or double mutations associated with them (e.g., Ser, 4/5; Leu 2/3). Fewer than 5% of the unexpected mutants had mutations outside the region encompassed by the oligonucleotide. The weight of the evidence suggests that unexpected mutations derive from impurities in the oligodeoxynucleotide pools and/or aberrant repair of the gapped ends.

Two codon-222 mutants (i.e., Gln and Ile) were not found after the screening described. To obtain these, a single 25-mer oligodeoxynucleotide was synthesized for each mutant, corresponding to the top oligonucleotide strand in Fig. 2. Each was annealed to the bottom strand of their respective oligodeoxynucleotide pool (i.e., pool A for Gln and pool D for Ile). This was ligated into cut pΔ222 and processed as described for the original oligodeoxynucleotide pools. The frequencies of appearance for single mutants obtained this way were 2/8 and 0/7 for Gln and Ile, respectively. To avoid this apparent bias the top strand was phosphorylated and annealed to its nonphosphorylated complementary pool. The heterophosphorylated cassette was ligated into cut pΔ222 and processed as before. The frequencies of appearance of Gln and Ile mutants were now 7/7 and 7/7, respectively.

DISCUSSION

When it is desirable to generate many mutations within a single site, the cassette mutagenesis method described has several advantages over methods that use single oligodeoxynucleotide mutagenesis in M13. (1) By synthesizing pools of oligodeoxynucleotides, less total oligodeoxynucleotide synthesis and purifi-

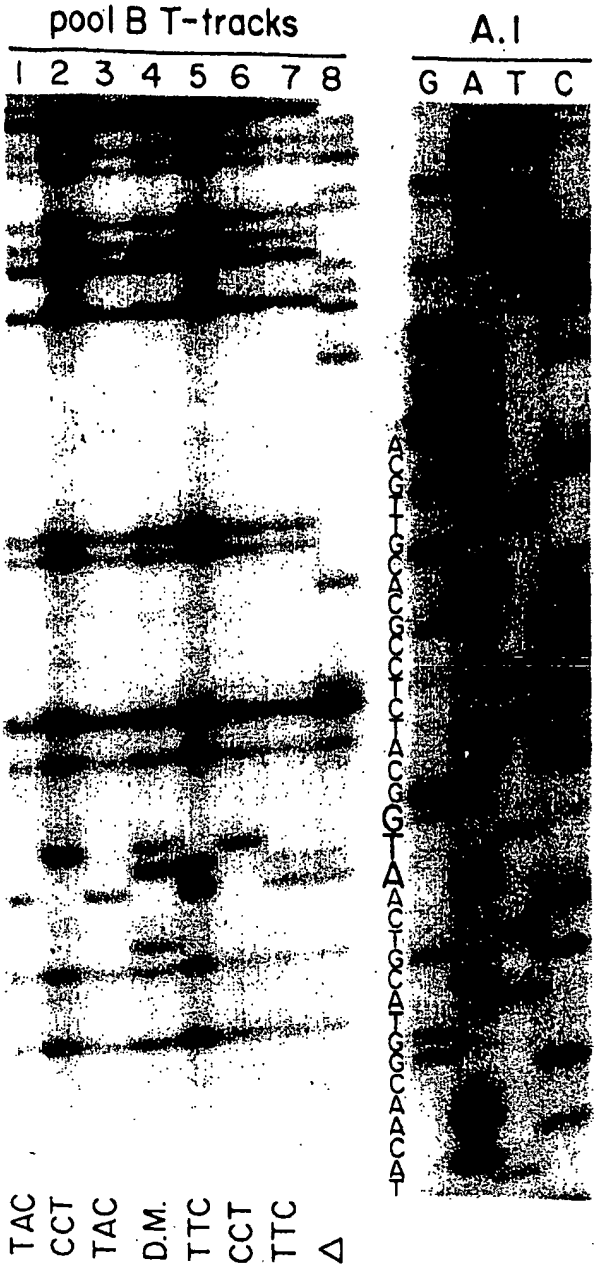

Fig. 3. Representative nucleotide sequence analysis of mutants obtained from pool B. Plasmid DNA was prepared and directly sequenced from the second-round transformants as described in MATERIALS AND METHODS, section c. Lanes 1–8 are single T sequencing lanes for clones B.1 to B.8. Adjacent to these lanes (lanes 9–12) is a reference sequence from clone A.1, which was a Met-222 isolate. The codons-222 predicted from the single lane analysis are shown below each track. Δ, deletion mutant, D.M.; double mutant.

TABLE I

Oligonucleotide pool organization and frequency of mutants obtained

Pool	Amino acids	Codon-222[a]	Frequency[b]
A	asp	GAT	2/25
	met	ATG	3/25
	cys	TGT	13/25
	arg	AGA	2/25
	gln	CAA	0/25
	unexpected mutants		5/25
B	leu	CTT	1/25
	pro	CCT	3/25
	phe	TTC	6/25
	tyr	TAC	5/25
	his	CAC	1/25
	unexpected mutants		9/25
C	glu	GAA	3/17
	ala	GCT	3/17
	thr	ACA	1/17
	lys	AAA	1/17
	asn	AAC	1/17
	unexpected mutants		8/17
D	gly	GGC	1/23
	trp	TGG	8/23
	ile	ATC	0/23
	ser	AGC	1/23
	val	GTT	4/23
	unexpected mutants		9/23

[a] Codons were chosen based on frequent use in the cloned subtilisin gene sequence (Wells et al., 1983).

[b] Frequency was determined from single track analysis by direct plasmid sequencing (Chen and Seeburg, 1985).

cation is required. (2) The mutagenesis event (i.e., ligation of the oligodeoxynucleotide cassette) is close to 100% efficient compared to in vitro heteroduplex synthesis in M13 (typically 1–30%). (3) Once the vector (e.g., pΔ222) is set up with flanking restriction sites, all manipulations (i.e., mutagenesis, sequencing, expression) can be performed in the same plasmid. Although it is possible to express some genes directly in M13 (Winter et al., 1982) this may not always be possible or desirable. (4) Due to the high efficiency of mutagenesis, mutants can be screened directly by nucleotide sequencing, which is more informative than probing with [^{32}P]oligonucleotides. (5) If the need arises other mutagenic cassettes can be readily inserted to alter other nearby codons.

The data in Table I show there is a bias in the frequency of mutants obtained from the pools. This probably resulted from unequal representation of oli-

godeoxynucleotides in the pool caused by unequal coupling of the particular trimers over the mutagenesis codon. Such a bias problem probably could be remedied by appropriate adjustment of trimer levels during synthesis to reflect equal reaction. In any case, generating mutants that were not picked up in the primary screen was readily achieved by synthesizing single oligodeoxynucleotides. The biased heteroduplex repair observed for the completely unphosphorylated cassette may result from the fact that position-222 is closer to the 5′ end of the upper strand than it is to the 5′ end of the lower strand (see Fig. 2). Because a break exists at the unphosphorylated 5′ ends and the mismatch bubble is at codon-222, excision repair of the top-strand break would more readily maintain a circularly hybridized duplex capable of replication. Consistent with this view is the fact that mutations directed from the top strand could be completely retained over mutations from the bottom strand by 5′ phosphorylation of the top strand.

Unexpected double mutants and deletion mutants appeared at a significant frequency (approx. 30%). This probably reflected impurities in the oligodeoxynucleotides, as in almost all cases the mutation appeared within the sequence governed by the oligodeoxynucleotide cassette. Oligodeoxynucleotide impurities can be a problem with any mutagenic procedure, and others have noted unexpected deletions (Osinga et al., 1983) and double mutations (Villafranca et al., 1983) using M13 protocols. We have recently applied the cassette mutagenesis protocol with success to two other sites (J. Wells and D. Powers, unpublished results). One of these sites employed oligodeoxynucleotide cassettes of 10 and 14 bp and were ligated into a 4-bp overhang and a blunt restriction site. The second site used 17- and 25-bp cassettes ligated into two 4-bp overhang sites. The frequency of unexpected mutants was significantly lower (<10%) in these experiments. This probably resulted from improved oligonucleotide purity and the fact that shorter oligonucleotide cassettes were employed.

The criterion for introduction of unique and nearby restriction sites to flank the target codon is facilitated by the fact that the number of commercially available restriction enzymes having sites not present in the gene of interest is generally large. A suitable nucleotide sequence computer search program simplifies the task of finding potential 5′ and 3′ unique flanking sites. A primary constraint is that any mutation introduced in creation of the restriction site must be silent with respect to the final constructed amino acid coding sequence. For a candidate restriction site 5′ to the target codon, a sequence must exist in the gene which contains at least all the nucleotides except one in the recognition sequence 5′ to the cut of the candidate enzyme. For example, the blunt cutting enzyme *Sma*I (CCC/GGG) would be a 5′ candidate if a nearby 5′ sequence contained NCC, CNC, or CCN. Furthermore, if N needed to be altered to C this alteration must leave the amino acid coding sequence intact. In cases where a permanent silent mutation is necessary to introduce a restriction site one might want to consider whether the mutation introduces a rarely used codon. A similar situation for *Sma*I would apply for 3′ flanking sites, except the sequence NGG, GNG, or GGN must exist. The criteria for locating candidate enzymes is most relaxed for blunt cutting enzymes and most stringent for 4-bp overhang enzymes. Flanking sites should obviously be chosen which cannot ligate themselves, so that ligation of the oligodeoxynucleotide cassette can be assured in a single orientation. A general search of the subtilisin gene has shown any codon can be set up for cassette mutagenesis, so that cassettes less than 30 bp and often less than 20 bp in length are required.

Coupling of pools of oligodeoxynucleotide trimers over the target codon using phosphotriester chemistry is a convenient way of synthesizing the indicated pools. However, defined duplex pools can be synthesized by monomer addition also. For example, the 20 codons can be grouped in oligonucleotide pools of two in such a way that two oligodeoxynucleotides differ by only a single base change (e.g., Met (ATG) and Ile (ATC) codons can be grouped). The oligonucleotide pool can be synthesized by adding an equal mixture of two nucleotides at the position of ambiguity (e.g., equal amounts of G and C). This pool can be combined with a complementary pool for two other codons [e.g., Leu (CTT) and Pro (CCT)]. Because heteroduplex cassettes ligate without difficulty, each mutagenic codon should be present at roughly 25% in the final pool of transformants. One may specifically target biased incorporation of the upper or lower strand pool by selective phosphorylation. In addition, the pools can be or-

ganized to facilitate screening by single-track nucleotide sequence analysis (e.g., a C track would distinguish members of the pool proposed for Met, Ile, Pro and Leu). In this way only 11 instead of 20 oligodeoxynucleotides need to be synthesized separately.

Matteucci and Heyneker (1983) reported a general method for introduction of randomized mutagenic DNA cassettes. The mutagenic cassette was inserted into an asymmetric gap created at a pre-existing restriction site by restriction followed by S1 digestion and idling back with DNA polymerase large fragment in the presence of limiting nucleotides. The method was applied to mutation of the spacer region between the Shine-Dalgarno sequence and the ATG. It has the advantage of precisely recreating the sequence at the points of ligation. However, due to the inefficiency of this construction (less than 50% contained inserts), clones not containing inserts and the wild-type background had to be distinguished from mutagenic inserts by hybridization and restriction analysis prior to sequencing.

While this work was in progress, Hui et al. (1984) reported preparation of 39 random triplet mutants by insertion between pre-existing restriction sites of an oligodeoxynucleotide cassette containing a fully randomized target trimer (i.e., a pool of all 64 possible triplets). This approach was especially well suited for studies of the effect on translation efficiency of different triplets preceding the initiation codon. However, if one is interested in obtaining only 20 specific triplets many unwanted and degenerate codons are present in such a large pool.

In summary, the method reported here is designed for specific codon mutagenesis. The method demonstrates the feasibility of introducing silent restriction sites which closely flank a target codon to allow highly efficient insertion of a mutagenic oligodeoxynucleotide cassette. Codons are organized in small pools to avoid exhaustive screening, and the pools are organized to facilitate rapid screening by single-track nucleotide sequence analysis. This method could also be applied to generate random mutations between the created restriction sites by insertion of an oligodeoxynucleotide cassette containing random mutations.

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